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Promoting microbial utilization of phenolic substrates from bio-oil

Kirsten Davis
Iowa State University, kirstdav@iastate.edu

Marjorie R. Rover
Iowa State University, mrrover@iastate.edu

Davinia Salvachúa
National Renewable Energy Laboratory

Ryan G. Smith
Iowa State University, rgsmith@iastate.edu

Gregg T. Beckham
National Renewable Energy Laboratory

See next page for additional authors

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Keywords
Pseudomonas putida KT2440, Lignin, Bio-oil, Emulsion, Phenols

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Authors
Kirsten Davis, Marjorie R. Rover, Davinia Salvachúa, Ryan G. Smith, Gregg T. Beckham, Zhiyou Wen, Robert C. Brown, and Laura R. Jarboe

REVISED MANUSCRIPT

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Kirsten Davis[a], Marjorie R. Rover[b], Davinia Salvachúa[c], Ryan G. Smith[b], Gregg T. Beckham[c], Zhiyou Wen[d], Robert C. Brown[b], Laura R. Jarboe[a*]

[a] K Davis, Prof. L. Jarboe*, Chemical and Biological Engineering, 4134 Biorenewable Research Laboratory, Iowa State University, Ames, IA 50011 United States. Phone: (515) 294-2319, Email: ljarboe@iastate.edu

[b] Prof. R. Brown, Dr. M. Rover, R. Smith, Bioeconomy Institute, Iowa State University, 1140 BRL Building, 617 Bissell Road, Ames, IA 50011 United States.

[c] Dr. D. Salvachúa, Dr. G.T. Beckham, National Bioenergy Center, National Renewable Energy Laboratory, Golden CO 80401 United States.

[d] Prof. Z. Wen, Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011 United States.

Abstract

The economic viability of the biorefinery concept is limited by the valorization of lignin. One possible method of lignin valorization is biological upgrading with aromatic-catabolic microbes. In conjunction, lignin monomers can be produced by fast pyrolysis and fractionation. However, biological upgrading of these lignin monomers is limited by low water-solubility. Here, we address the problem of low water-solubility with an emulsifier blend containing approximately 70 wt% Tween® 20 and 30 wt% Span® 80. *Pseudomonas putida* KT2440 grew to an optical density (OD_{600}) of 1.0±0.2 when supplied with 1.6 wt% emulsified phenolic monomer-rich product produced by fast pyrolysis of red oak using an emulsifier dose was 0.076±0.002 g emulsifier blend per g of phenolic monomer-rich product. This approach partially mitigated the toxicity of the model phenolic monomer *p*-coumarate to the microbe, but not benzoate or vanillin. This study provides a proof of concept that processing of biomass-derived phenolics to increase aqueous availability can enhance microbial utilization.

Keywords: *Pseudomonas putida* KT2440; lignin; bio-oil; emulsion; phenols

Introduction

Recent models indicate that the economic viability of the biorefinery concept relies on the valorization of lignin [15,44]. Even though lignin is a highly abundant biopolymer, it is currently slated to be used primarily for heat and power as a low value by-product of pioneer biofuel production facilities [54]. Aromatic-catabolic soil microbes are a promising route to add value to the lignin fraction of biomass due to their ability to catabolize many lignin-derived phenolic monomers and convert these molecules into renewable fuels and chemicals [9,1]. Among others, *Pseudomonas putida* KT2440 has emerged as a promising platform organism for such applications [42,36,28,70]. For example, *P. putida* can utilize a broad range of aliphatic and aromatic compounds as a sole carbon and energy source [51,65].

The number of reports demonstrating native and engineered microbial biocatalysts for biological lignin conversion has increased substantially in recent years [9,65,31]. Exemplary products include vanillin [63], muconic acid [70,8,35,69], fatty acids [78,38], polyhydroxyalkanoates [36], aromatic dicarboxylic acids [41], 2-pyrone-4,6-dicarboxylic acid [40], and lactic and pyruvic acids [29]. Many of the studies to date that have reported high titers, rates, and yields, however, have utilized model compounds due to the difficulties associated with achieving high yields of bio-available aromatic monomers from lignin to date. Even in model compound studies, substrate solubility in water is a substantial challenge [64].

Microbial modification of lignin requires some sort of processing to both separate the lignin from the other biomass fractions and then depolymerization of the lignin. A variety of methods exist for achieving these goals [49,14]. One approach is fast pyrolysis, which has the advantages of being rapid and low cost [55,73,23]. Fast pyrolysis of biomass produces several product streams:

a heavy ends stream which is rich in sugars and water-insoluble phenolics, bio-char which is collected via cyclones and can be used as a soil additive, and lastly, a light ends aqueous stream rich in acetate (Fig. 1) [66,4,11]. The water-insoluble phenolic oil stream is mainly composed of lignin-derived products, including monophenols such as phenol and syringol, in addition to higher molecular weight phenolic oligomers [4], with its composition being impacted by the pyrolysis conditions [62]. The insolubles are separated from the unrefined sugars with a simple aqueous liquid-liquid extraction [61,72].

For the fast pyrolysis-based biorefinery, the addition of value to lignin requires upgrading the components of this phenolic oil. However, its low water solubility is problematic for traditional aqueous-phase bioconversions. The problem of low substrate solubility has previously been addressed through the use of emulsifiers to improve degradation of hydrocarbons, such as naphthalene and pyrene, by *Pseudomonas* species [30]. Emulsifiers increase the concentration and bioavailability of poorly soluble molecules by forming micelles in emulsions [37,17]. These compounds are adsorbed at the oil/water interface, lowering the interfacial surface tension [39], but because the resulting mixtures are non-equilibrium systems, they do eventually undergo breakdown processes [39,47]. Span® and Tween® emulsifiers, employed in this study, are amphipathic (i.e. contain both hydrophilic and hydrophobic functionality), non-ionic surface-active surfactants forming strong hydrogen bonding with water [50]. Non-ionic surfactants are appealing for industrial processes because of their relatively simple production process, which can utilize a variety of feedstocks at a fairly low cost [2].

Emulsification and dispersion methods can be designed to tune the stability and rheology of the resulting mixtures [77]. Methods such as colloidal milling, homogenization, and ultrasonication, result in relatively stable mixtures [46]. Contrastingly, the use of vortex mixing

produces mixtures containing isolated and sparse droplets with sustained release. For example, emulsions promoting the sustained release of adjuvants and antigens were consistently and reliably produced by vortexing [20]. Here, we have used vortex mixing to promote the slow release of the phenolic monomers into the aqueous cultivation medium.

The goal of this research is to investigate emulsification as a means of increasing the biological availability of aromatic monomers produced from lignin to microbial biocatalysts for the production of value-added products. Not only will this potentially enable the production of value-added products and specialty chemicals from lignin, this work also provides insight on how to mitigate the toxicity of these phenolic compounds to microbial biocatalysts.

Experimental

Heavy Ends Bio-oil Production

Red oak (*Quercus rubra*) was procured from Wood Residual Solutions of Montello, WS. As-received biomass was passed through a 60 hp hammer mill with a 3 mm screen, resulting in a particle size range of approximately 200 micron to 3 mm. The moisture content of the red oak was approximately 10 wt%. Bio-oil was produced in a fluidized bed reactor and collected in a bio-oil recovery system that separates the bio-oil into the heavy ends and light ends (Fig. 1). The heavy ends stage collects the viscous, high-boiling-point compounds referred to collectively here as heavy ends bio-oil, and was used in this research [61]. Details of the pyrolyzer and recovery system can be found in Rover et al [62] and Pollard et al [45].

Liquid-liquid Extractions

The heavy ends bio-oil was subjected to an aqueous liquid-liquid extraction (Fig. 1) to separate the water-soluble components, such as sugars and acetate, from the water-insoluble phenolics, as detailed by Rover et al [62]. Briefly, a 1:1 w/w mixture of heavy ends bio-oil:water was mechanically stirred using a drill press equipped with a stainless steel open paddle for 10 – 15 min during bio-oil production, placed on a shaker table (MaxQ 2506, Thermo Scientific®, Hanover Park, IL) for 30 min at 250 motions min^{-1} and centrifuged (accuSpin™ 1R, Thermo Scientific®, Hanover Park, IL) at 2,561g force for 30 min. The water-soluble unrefined sugars were decanted from the water-insoluble phenolics. The water-insoluble phenolic fraction was centrifuged and decanted again to remove any remaining unrefined sugar solution.

Secondly, an extraction of lower molecular weight aromatics from the water-insoluble phenolic oil was accomplished by liquid-liquid extraction with toluene (Fig. 1) [3]. An equal mass of toluene was added to the water-insoluble phenolic oil and stirred with a hand drill equipped with a stainless steel open paddle for 10 min. The toluene-soluble extract was decanted from the toluene-insoluble fraction. The toluene was then recovered from the toluene-soluble extract via evaporation utilizing a Heildolph Hei-Vap Precision rotary evaporator (Fisher Scientific, PA) at 77 mbar and 40 °C. The product from the toluene extraction after evaporation, referred to here as phenolic monomer-rich product, was used in the emulsions for this work.

A gas chromatograph with a flame ionization detector (GC-FID) was used for identification of components of the phenolic monomer-rich product using a 430 (Bruker Corporation, Bruker Daltonics, Inc. Fremont, CA) GC-FID. The column was a 1701 capillary, 60 m in length, 0.25 mm inner diameter, with a 0.25 mm film thickness (Phenomenex, Inc., Torrance, CA). Galaxie

Chromatography Data System version 0.9.302.530 (Bruker Corporation, Bruker Daltonics, Inc., Fremont, CA) was the operating system. The carrier gas was helium (99.9995%) with a constant flow rate of 1.0 mL min^{-1} and the helium make-up was 25 mL min^{-1}, with hydrogen flow at 30 mL and an air flow of 300 mL min^{-1}. The oven was programed to be held for 4 min at 45 °C and ramped at 3 °C min^{-1} to 235 °C and held for 10 min. The sample volume was 1 µL with a split ratio of 1:45. Peak identification was based on calibration standards purchased from Fisher Scientific (Thermo Scientific, Hanover Park, IL) [60].

Phenolic Monomer-rich Product Emulsification

Two emulsifiers were used in this study: Span® 80, a non-ionic surfactant, and Tween® 20, a non-ionic detergent. Span® 80 is widely used in food and pharmaceutical applications. Tween® 20 is a food additive.

To determine the required Hydrophile-Lipophile Balance (HLB) values, an expression of the size and strength of the hydrophilic (polar) and lipophilic (non-polar) groups of the emulsifier [24], test emulsions were made with different emulsifier blends. When two or more emulsifiers were blended, the HLB value was calculated using the weight percentage of each emulsifier multiplied by the HLB value of the neat emulsifier. The individual HLB values were then summed for all emulsifiers to calculate the HLB for the blend. Equation 1 [24] was used to calculate the HLB values.

$$Emulsifier\ \#1: \quad (wt\%\ emulsifier\ \#1)\ x\ (neat\ HLB\ emulsifier\ \#1)\ =\ X \quad [Eq.1]$$

$$Emulsifier\ \#2: \quad (wt\%\ emulsifier\ \#2)\ x\ (neat\ HLB\ emulsifier\ \#2)\ = Y$$

$$HLB\ of\ the\ Blend\ =\ X + Y$$

The target HLB range for the blends can be determined either from known HLB values for the constituents of the material to be emulsified or from assessment of the amount of product emulsified, any indication of separation, and clarity of the resulting emulsion. The test emulsions were comprised of 1.0 g phenolic monomer-rich product, 10 g water and 0.15 g of the emulsion blend and were vortexed. Each emulsifier blend was evaluated in terms of the amount of phenolic monomer-rich product emulsified, any emulsion separation due to Oswald ripening, flocculation, coalescence, creaming, and sedimentation. Lastly, the clarity of the mixture was evaluated [12]. The control was 1.0 g of the phenolic monomer-rich product vortexed with 10 g water without emulsifiers.

The mixture for microbial utilization studies were prepared by first adding deionized (DI) water to a 50 mL centrifuge tube, followed by the phenolic monomer-rich product. The polar emulsifier (Tween® 20) was added, followed by the nonpolar Span® 80. The relative amount of emulsifiers, water and phenolic monomer-rich product varied according to experimental parameters. The mixture was vortexed for 5 minutes. The amount of emulsifier is reported as the grams of emulsifier blend per gram of phenolic monomer-rich product. The resulting liquid mixture was decanted away from the residual phenolic monomer-rich product that did not emulsify. This residual material was dried at 80 °C and weighed to determine the mass of phenolic monomer-rich product that was not emulsified.

Total Phenolics Determination

Modified micro-scale methodology for the Folin-Ciocalteu (FC) method was used to determine total water-soluble phenolic monomers present in the monomer-rich product using a procedure outlined by Rover and Brown [58]. Briefly, a 20 µL monomer-rich product sample

(prepared with 0.15 - 0.30 g of monomer-rich product diluted with 5 mL deionized water then diluted to 50 mL mark with deionized water and filtered with 0.45µm Corning syringe filter), a blank consisting of deionized water, and Gallic acid calibration standards were each placed in 2 mL polystyrene cuvettes. Deionized water (1.58 mL) was added, followed by 100 µL FC reagent. The solution in each cuvette was mixed thoroughly by pipetting, and each were incubated for 1-8 min. This was followed by the addition of 300 µL of sodium carbonate solution and incubation for 2 h at room temperature. The sample absorbance was measured at 765 nm with 1 cm cells and a 1.5 nm bandwidth with a Varian Cary 50 UV-visible Spectrophotometer (Agilent Technologies Inc., Santa Clara, CA) using Cary WinUV (Agilent Technologies Inc., Santa Clara, CA) Simple Reads module software. The sodium carbonate solution was made by dissolving 200 g anhydrous sodium carbonate in 800 mL water and brought to a boil. After cooling, a few crystals of sodium carbonate were added. The solution was stored for 24 h at room temperature and then filtered with a Whatman #42 filter paper and water was added to make 1.0 L. A minimum of five trials were performed for each monomer-rich product sample.

Media Preparation

Microbial growth media consisted of modified M9 medium, prepared according to Johnson et al., which contains no carbon source [29]. Pure monomers (benzoate or *p*-coumarate), in the emulsified or non-emulsified state, or emulsified phenolic monomer-rich product were added as carbon source. The carbon source concentration is reported as the weight percent of phenolic monomer in growth medium according to Equation 2. The estimated concentration of total emulsifier in growth medium is reported according to Equation 3. It was assumed that all of the emulsifier was recovered in the growth medium.

$$\frac{\text{Mass}_{\text{phenolic monomer-rich product added}} - \text{Mass}_{\text{residual phenolic monomer-rich product}}}{\text{Total volume of growth medium} * \text{Density of growth medium}} * 100\% \text{ [Eq. 2]}$$

$$\frac{\text{Mass}_{\text{emulsifier added}}}{\text{Total volume of growth medium} * \text{Density of growth medium}} * 100\% \text{ [Eq. 3]}$$

The density of the culture medium was assumed to be 1.0 g/mL. The resulting media was adjusted to a pH of 7.0 with sodium hydroxide and sterilized with a 0.2 µm pore size syringe filter.

Microbial Cultures

Bacterial cultures were grown in 20 mLs of growth medium with 250 mL shake flasks at 30 °C, 200 RPM, and initial pH of 7.0. Single colonies of *P. putida* KT2440 were pre-cultured overnight in 10 mL of Luria Broth. Pre-cultures were centrifuged (Thermo Fisher Scientific Sorvall Legend XTR) at a relative centrifugal force of 3,488 x g for two minutes and re-suspended in modified M9. These washed cells were diluted with growth media to an optical density of 0.05 at 600 nm.

Samples were taken regularly during growth until cells reached stationary phase. At each time point, samples were centrifuged (Eppendorf 5424 Microcentrifuge) at a relative centrifugal force of 21,130 x g for five minutes, the supernatant was removed, and the cells were re-suspended in DI water for measurement on a spectrophotometer at 600 nm (Thermo Scientific, Genesys20). Unless stated otherwise, p values were calculated using a pair-wise Student's two-tailed, equal variance t-test.

Results and Discussion

Emulsion Studies

Selection of emulsifier blend. Microbial biocatalysts, such as *P. putida*, are an attractive method of upgrading the biomass-derived phenolic monomer-rich product to biorenewable fuels and chemicals. Unfortunately, this stream has a very low water solubility thus restricting the availability of the phenolic monomers in the cultivation media. Specifically, assessment of water-soluble phenolic monomers in the monomer-rich product were determined utilizing the modified FC method [57]. These measurements indicated that only 316±1 mg/L of the phenolic monomer-rich product was water-soluble. We proposed to address this low solubility by emulsifying the phenolic monomer-rich product, while also enabling the slow release of the phenolics from the emulsion for microbial utilization.

Emulsion stability is related to different parameters such as oil content, emulsifier content, pH, temperature, and HLB, a metric of the size and strength of the hydrophilic (polar) and lipophilic (non-polar) groups of the emulsifier [24]. While there is not a single parameter that can explain the stability of an emulsion [50], HLB values are a reliable predictor of how an emulsifier will behave in mixtures and can be used as a guide in selection of emulsifiers. Specifically, the HLB value of the emulsifier or emulsifier blend should be the same as the HLB value of the material to be emulsified [24]. The HLB value for the complex phenolic monomer-rich product is not known, but the phenolic monomers benzene, ethyl benzoate, and styrene all have HLB values between 13 and 15 [24]. Thus, we expect that the HLB value required for emulsification of the phenolic monomer rich product will be within the high end of the 8-18 range generally considered to be suitable for an oil-in-water emulsifier [2,21]. Tween® 20 (hydrophilic) and Span® 80

(lipophilic) were selected as model emulsifiers due to their known lack of biological toxicity, lower cost [2], and their HLB values of 16.7 and 4.3, respectively [24].

Emulsification of 1.0 g of phenolic monomer-rich product in 10.0 g of DI water was assessed through the use of 0.15 g of pure Tween® 20, pure Span® 80, and five blends of Tween® 20 and Span® 80 (Fig. 2). The HLB values of the pure emulsifiers and the various blends ranged from 4.30 to 16.7. The test emulsion with an HLB value of 13.2 (sample 4) was chosen as the best candidate based on clarity. The use of pure Span ® 80 (sample 8) was comparable to the no-emulsifier control (sample 1), in terms of the lack of solubilization of the phenolic monomer-rich product. Test emulsions with a calculated HLB values of 15.0 and greater (samples 2 and 3) and of 10.2 and lower (samples 5, 6 and 7) were deemed unsuitable due to the cloudy appearance.

While emulsification of the phenolic monomer-rich product was our primary criteria in selection of emulsifier blend, it was also important that the mixture would break down over time to release the monomers for microbial utilization. Stability was evaluated by visually judging the amount of sedimentation, flocculation, and creaming of the test emulsions over a several week time period. Emulsions 2 and 3 appeared to be too stable, as no phenolic monomer-rich product came out of the emulsion, while for samples 4 and 5 the phenolic monomer-rich product was partially released from the emulsion, as evidenced by collection on the bottom of the beakers. The remaining test emulsions, samples 6-8, did not have enough oil incorporated into the emulsions to assess stability. Because sample 4 (71.5 wt% Tween® 20, 28.5 wt% Span® 80) was acceptable both in terms of clarity and stability, it was chosen for further characterization.

Emulsified phenolic monomer-rich product contains monomers suitable for microbial utilization.

The goal of emulsification of the phenolic monomer-rich product is to increase the availability of the lignin-derived monomers in the aqueous cultivation media. A calibrated GC/FID was used to determine the concentration of compounds that were present in the emulsion generated using the 70:30 wt% Tween® 20, Span® 80 blend and to also identify compounds not detectable in the emulsion (Table 1, Fig. S1). Many of these compounds were also detected in the aqueous sugar wash solution produced in the same bio-oil recovery and processing framework [11].

The most abundant phenolic monomer was acetosyringone, which has been characterized as a signaling molecule in plant-pathogen interactions [5] and as an inhibitor of *Saccharoymyces cerevisiae*, a commonly-used fermentation organism [5,34]. Microbial species such as *Pseudomonas* have been previously described to utilize many of the other monomers, such as phenol [19,7,68,35], cresol [7,35], styrene [43], xylene [67], ethylbenzene [67], and 3,4-dimethylphenol [68]. Other compounds, such as 2,6-dimethoxyphenol [74], 4-vinylguaiacol [32], and eugenol [48], have been reported to be subjected to biological modification, but it is not clear if the reaction products are funneled into central metabolic pathways. Thus, the emulsified phenolic monomer-rich product contains at least some species that can be utilized by microbial biocatalysts.

Microbial Studies

Microbial utilization of emulsified phenolic monomers.

The emulsified phenolic monomer rich product contains many phenolic monomers that have previously been demonstrated as substrates for microbial species, such as *Pseudomonas*. The microbial production of industrial fuels and chemicals from lignin-derived monomers requires funneling of these monomers into central metabolism. Here, we used microbial growth as an

indicator of utilization of these monomers through central metabolism. Specifically, utilization of the phenolic monomers by *P. putida* was assessed based on the maximum OD_{600} observed over a 72-hour period (Fig. 3, Fig. S2). It should be noted that only a portion of the phenolic monomer rich product is emulsified, as accounted for with Equation 3. Thus, Fig. 3 reports the emulsifier dosage used for the emulsification process as well as the final concentrations of the emulsifier and the phenolic monomer rich product in the growth medium.

As shown in Fig. 3a, the 70:30 blend of Tween® 20 and Span® 80 with the phenolic monomers allowed *P. putida* to achieve an OD_{600} of 0.87 ± 0.04, a 4-fold higher value ($p = 0.005$) than the maximum OD_{600} observed when the growth medium contained only emulsifier and no phenolic monomers. This increased growth in the presence of the emulsified phenolic monomer indicates that the lignin-derived monomers are being used to produce the central metabolic intermediates needed for microbial growth. The concentration of each lignin-derived monomer in the growth medium will vary not only with the targeted concentration of phenolic monomer-rich product added to the medium, but also with the emulsification process. At an emulsifier dose of 0.205±0.008 g per g of monomer-rich product and 0.24 wt% phenolic monomer-rich product (Fig 3a bar 1), each lignin-derived monomer in the growth medium should be present at roughly 13% of the concentrations in the emulsion (Table 1a). For example, the growth medium should contain approximately 80 mg/L of acetosyringone. Our observed utilization of the emulsifiers as sole carbon source is consistent with previous reports involving Triton X-100 and Brij 30 [17]. The fact that there was no significant difference in maximum OD_{600} in the media containing only the Tween® 20/Span® 80 mixture and the media containing only Tween® 20 suggests that *P. putida* is able to use the Tween® 20, but not Span® 80, as a carbon source. Note that data are not shown

for media in which Span® 80 is the only potential carbon source, because it was insoluble at the experimental concentration.

To assess the benefit of using an emulsifier blend, the phenolic monomer-rich product was emulsified with just Tween® 20 or just Span® 80, similar to samples #2 and #8 in Fig. 2. Microbial growth on these emulsions was much lower than the growth observed using the 70:30 blend (Fig. 3A), validating the tuning of the emulsifier blend described above.

Microbial growth scales with emulsifier concentration.

As shown above, emulsification of the phenolic monomer rich product with a 70:30 blend of Tween® 20 and Span® 80 promotes microbial growth on this stream. This growth is a proxy for funneling of the phenolics into central metabolic intermediates. For the experiments described above, 0.205±0.008 g of the emulsifier blend was used per g of phenolic monomer rich product. Given that only a portion of the phenolic monomer rich product is solubilized in the emulsion process (Table 2), the resulting concentration of emulsifier in the growth medium (0.28 wt%) was roughly equal to the concentration of phenolic monomer in the growth medium (0.24 wt%). However, since the emulsifier contributes to the process cost, the emulsifier should be used as sparingly as possible.

Decreasing the amount of the emulsifier blend used resulted in a decrease in the amount of phenolic monomer recovered in the emulsion (Table 2). The emulsified product was then added to microbial growth medium such that the phenolic monomer concentration was maintained at 0.24 wt%, resulting in varying concentrations of emulsifier in the growth medium (Fig. 3b). Despite the constant concentration of emulsified phenolic monomer-rich product, the maximum OD_{600} reached by *P. putida* decreased as the emulsifier dosage decreased (Fig. 3b). Note that emulsions made with lower doses of emulsifier still promoted growth, although the maximum OD_{600} was

significantly decreased ($p < 0.05$). As described above, *P. putida* is able to utilize the emulsifier itself as sole carbon source (Fig 3a), and thus the decrease in maximum OD_{600} could be due in part to the decrease in total carbon available to the *P. putida.* Decreasing the amount of emulsifier used may also change the nature of the emulsion, leading to stability issues over time. For the rest of the studies described here, the emulsifier blend was dosed at 0.08 g of emulsifier blend per g of phenolic monomer-rich product.

Monomer emulsification can provide protection from growth inhibition.

Phenolic monomers and other organic molecules produced during biomass degradation are known to inhibit the growth of bacteria and yeast [13,27]. Therefore, the effect of the concentration of the phenolic monomer-rich product on cell growth was investigated (Fig. 4, Fig. S3). When the concentration of phenolic monomer-rich product in the growth medium was increased from 0.24 wt% to 0.80 wt%, there was a significant increase of over 4.5-fold in the maximum OD_{600}. In the range of 0.4-1.6 wt% phenolic monomer-rich product, no significant differences in maximum OD_{600} were observed, though the maximum OD_{600} did show a downward trend as the concentration of phenolic monomer was increased from 0.80 to 1.60. The concentration of phenolic monomer-rich product was not increased past 1.6 wt% due to the amount of phenolic monomer-rich product that can be emulsified.

Because the emulsifier dosage was maintained at a constant 0.08 g/g for these experiments, the amount of emulsifier in the media ranged from 0.03 - 0.82 wt%. Although *P. putida* was observed to use the emulsifier blend as sole carbon source (Fig. 3a), the maximum OD_{600} was significantly higher in the presence of emulsified monomer-rich product relative to the corresponding emulsifier-only control within the range of 0.24 – 1.06 wt% monomer-rich product.

These results support the conclusion that the phenolic monomers are being used to support biomass production.

The primary goal of emulsifying the phenolic monomer-rich product is to make these compounds available to the biocatalyst in the aqueous phase. However, it is possible that the emulsification also helps to protect *P. putida* from inhibition by these monomers. It is difficult to test the effect of the emulsion against a non-emulsified control because the phenolic monomer-rich product is not soluble in water (Fig. 2). Coumarate and benzoate were selected here as model water-soluble aromatic monomers known to be utilized by *P. putida* KT2440, though they are unlikely to be present in the pyrolysis-derived material. Vanillin is a methoxylated aromatic aldehyde that has also been reported to be utilized by *P. putida* KT2440 [70] and has been detected in lignin depolymerized by fast pyrolysis [71].

During growth on non-emulsified *p*-coumarate, the maximum OD_{600} dramatically decreased from 1.06-1.60 wt%, indicating growth inhibition due to *p*-coumarate toxicity (Fig. 5a). However, a maximum OD_{600} of 3.39±0.02 was observed when cultures contained 1.60 wt% emulsified *p*-coumarate, indicating that the emulsified *p*-coumarate is not as inhibitory as the non-emulsified form. At 0.8 wt% *p*-coumarate, there was also an increase in maximum OD_{600} for the emulsified *p*-coumarate relative to the non-emulsified *p*-coumarate. Although some of the improvement in the emulsion trials could possibly be attributed to the additional carbon provided by the emulsifiers, this benefit should only result in, if any, a small increase in maximum OD_{600}. As shown in Fig. 4, the same combination of Tween® 20 and Span® 80, but a higher total emulsifier concentration relative to the *p*-coumarate trials, resulted in a maximum OD_{600} of only 1, much lower than the values observed during growth in the presence of *p*-coumarate. Therefore, the difference of more than 3 OD_{600} units is unlikely to be due to utilization of the emulsifiers.

P. putida KT2440 growth on varying concentrations of the non-emulsified pure phenolic monomer *p*-coumarate can be compared to previous studies. A report on tolerance mechanisms to *p*-coumarate indicated that the growth rate of *P. putida* KT2440 was negatively impacted at concentrations of 0.49 wt% *p*-coumarate and higher when supplemented with 0.2% glucose [10]. However, in our study, the maximum OD_{600} was greatest at 1.1 wt% *p*-coumarate, but decreased to almost 0 at 1.6 wt% *p*-coumarate. The slightly increased tolerance to *p*-coumarate in the current study may be due to the difference in carbon source availability.

Unlike the clear improvement in growth on *p*-coumarate, the maximum OD_{600} was not significantly different when cells were provided with 0.1 – 1.1wt% emulsified benzoate relative to non-emulsified benzoate. Although a significant difference in the maximum OD_{600} values during growth with 1.6 wt% benzoate was observed, both of the values were quite low, indicating that this concentration of benzoate is toxic to the cells. When we varied the concentration of non-emulsified pure benzoate to determine tolerance, growth was consistent with a previous study on benzoate stress response [53]. In the previous study, *P. putida* KT2440 was cultivated on pure benzoate as a sole carbon source. The growth rate was negatively impacted when benzoate concentrations were at or above 0.86 wt% [53]. Similarly, in our study, the maximum optical density was negatively impacted at concentrations of 1.06 wt% benzoate and higher (Fig. 5b).

Surprisingly, emulsification of vanillin was observed to significantly decrease the maximum OD_{600} at low vanillin concentrations and did not lead to any significant differences in maximum OD_{600} at higher concentrations (Fig. 5c). At 0.24 wt% vanillin, there was substantial variability between technical replicates, particularly in regards to lag time. However, extension of the observation period to 150 hrs still leads to the conclusion that there was no significant

difference between maximum OD_{600} values when cells were provided with emulsified or non-emulsified vanillin. The low growth in the presence of 0.24 wt% non-emulsified vanillin is consistent with previously reported inhibitory vanillin concentrations for commonly-used fermentation organisms [25].

Thus, substantially different outcomes were observed regarding the impact of emulsification of three model phenolic monomers on utilization of these monomers by *P. putida*. Emulsification promoted growth in the presence of p-coumarate, had no impact on growth in the presence of benzoate, and had a negative impact on growth in the presence of vanillin. It has been previously reported that emulsification of the cyclic terpene D-limonene, the aromatic unsaturated aldehyde cinnamaldehyde, and the monoterpoid phenol carvacrol with Tween® 20 and glycerol mono-oleate actually increased the toxicity of these compounds to *Escherichia coli*, *Lactobacillus delbrueckii*, and *S. cerevisiae* [16]. Thus, emulsification is not a universal strategy to provide protection to microbes from inhibitory compounds present in the growth media.

These three representative monomers differ substantially in their toxicity to *P. putida* (Fig. 5). For example, substantial growth was observed in the presence of 1.06 wt% (65 mM) *p*-coumarate but not 1.06 wt% (74 mM) benzoate. Vanillin toxicity was much more severe, with very little growth observed in the presence of 0.24 wt% (16 mM). Some studies characterizing the growth of *Pseduomonas* species on these compounds use a monomer concentration of approximately 5 mM [52], well below the toxicity limits observed here. The differing toxicity of these molecules is similar to previous observations that aldehydes, such as vanillin, tend to inhibit microbial growth at lower concentrations relative to organic acids [75,76]. However, assessment of these three compounds as inhibitors of the osmophilic yeast *Zygosaccharomyces rouxii* concluded that *p*-coumaric acid was less inhibitory than sodium benzoate and vanillin, but

observed roughly equal effects of benzoate and vanillin [56]. The relative toxicity of these types of molecules is often attributed to differences in hydrophobicity [6,75,76], non-polar surface area [6] and positioning of electron-withdrawing and electron-donating functional groups [22].

Process Feasibility

Here, we used a blend of the emulsifiers Tween® 20 and Span® 80 to disperse the phenolic monomer-rich product into the aqueous microbial growth medium. Characterization of other emulsifier blends or other emulsification techniques may identify emulsification methods that are more effective and/or have a lower cost. It is possible that the emulsifiers could be recycled, but this has not been investigated here. The utilization of Tween® 20 by the microbial biocatalyst is concerning in the context of process cost and emulsifier recycling, further characterization may identify a more suitable replacement.

Regardless of the substrate type, a relatively low substrate concentration in a cultivation process will inherently lead to low concentrations of the metabolic product, resulting in a relative increase in product separation costs. This problem of substrate toxicity, and the associated increase in product separation costs, is widespread in the biorenewables field [26]. It is demonstrated above that the emulsified phenolic monomer-rich stream can be provided at concentrations up to 1.60 wt% without negatively impacting microbial growth (Fig. 4). Higher concentrations were not tested, but could possibly be achieved with changes to the emulsification method.

The biomass depolymerization and fractionation process used here involves two liquid-liquid extraction steps (Fig. 1). First, water is used to separate the water-soluble sugars from the phenolic-insoluble phenolic oil. There are a variety of potential applications for this bulk phenolic oil, including coal replacement [59], production of gasoline and diesel [18,60], and use in resins [33]. Here, we performed a second extraction on the bulk phenolic oil, using toluene as the solvent,

to separate the high-molecular weight phenolics from the phenolic monomer-rich product. The toluene was then removed via distillation with 99.8% recovery (*data not shown*). Other methods of removing the high-molecule weight phenolics from the bulk phenolic oil may be more effective, have lower cost or use less toxic solvents; these have not been investigated here. However, our demonstration that emulsification of this stream of depolymerized biomass promotes microbial utilization should be applicable across a wide array of biomass processing strategies. Technoeconomic analysis of this process, which is not presented here, could guide these types of decisions.

Conclusions

This work demonstrates that microbial utilization of the phenolic monomer-rich product of biomass fast pyrolysis is promoted by processing of this stream to increase the concentration of the monomers in the aqueous growth medium. Specifically, we were able to provide microbial cultures with more than 1.0 wt% emulsified material without negatively impacting growth. The emulsifier blend used here seems to not only increase the aqueous concentration of a variety of phenolic monomers (Table 1), but may also provide protection against the inhibitory effect of some of these monomers.

Here, we used *P. putida* KT2440 as a model microbial biocatalyst, but this approach can be generalized to other microbial species. The optimum emulsion formulation consists of 70:30 (by mass) Tween® 20 to Span® 80. The amount of emulsifier blend added to the phenolic monomer-rich product had a direct impact on the amount of phenolic monomer-rich product emulsified and on the microbial growth, and we did observe evidence of microbial utilization of the emulsifiers, particularly Span® 80. Additional emulsion work, identification of specific compounds in pyrolysis phenolic oil utilized by *P. putida* KT2440, and other suitable microbes

are obviously needed before this approach could be used in an economically viable process. However, these results provide valuable proof of concept towards the microbial utilization of depolymerized lignin in a biorefinery concept and contribute to a better understanding of the influence of emulsions to enhance biological availability of bio-oil water insoluble constituents for fermentation.

Conflicts of interest

There are no conflicts of interest.

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Figure Legends

Fig. 1. Schematic of the fast pyrolysis reactor and the heavy and light ends of bio-oil recovery. The heavy ends is subjected to liquid-liquid extraction using water as the solvent during bio-oil production [45,62]. The products from the liquid-liquid extraction are unrefined sugars and water-insoluble phenolic oil. The cyclones collect the biochar while the light ends contain the water and is rich in acetate. The heavy ends water-insoluble phenolics are subjected to liquid-liquid extraction utilizing toluene as the solvent. Following removal of the toluene, the phenolic monomer-rich product is subjected to microbial utilization.

Fig. 2 Selection of a blend of approximately 70:30 wt% of Tween® 20 and Span® 80 (sample number 4) as optimum for emulsification of the phenolic monomer-rich stream. Each emulsion consisted of 1.0 g phenolic monomer-rich product and 10.0 g DI water. For samples 2 - 8, 0.15 g of total emulsifier was added. HLB values were calculated according to Eq 1. Each blend was assessed on the basis of clarity immediately after mixing. Stability of the mixture over the course of several weeks was assessed for some blends, as indicated.

Fig. 3. Emulsification enables microbial utilization of the phenolic monomer-rich product.
a). Phenolic monomer-rich product emulsions made with an approximately 70:30 combination of Tween® 20 and Span® 80 promoted higher cell growth than those without phenolic monomer-rich product. Emulsions with just Span® 80 were not assessed due to low solubility. Phenolic monomer-rich product emulsions made with only Tween® 20 or only Span® 80 supported significantly lower growth than the 70:30 blend. The letters above the bars indicate statistically significant groupings of the maximum OD_{600} values ($p<0.05$), as shown in the bar graph. As not all of the phenolic-monomer rich product is incorporated into the emulsion, the relative abundance

of emulsifier and phenolic monomer in the growth medium differs from the emulsifier dose. Growth curves are provided as Fig. S2.

b). Growth on phenolic monomer-rich product emulsions is directly related to total emulsifier concentration. All emulsions were made using a emulsifier blend containing 66.3-73.9 Tween® 20 : 26.1-33.7 Span® 80. The amount of emulsified product added to the growth medium was varied so that samples contained 0.24 wt% monomer-rich product.

P. putida KT2440 was grown at 30 °C and 200 RPM and initial pH of 7.0. Values are the average of at least two biological replicates with the error bars representing one standard deviation. The reported value for the concentration of monomer-rich product in the growth medium reflects the fact that not all of the monomer-rich product is solubilized (Table 2).

Fig. 4. Emulsified phenolic monomer-rich is utilized at concentrations of at least 1 wt%. *P. putida* KT2440 was grown for 70 hours at 30 °C and 200 RPM and initial pH of 7.0 and a emulsifier dosage of 0.08 g of emulsifier blend per g of phenolic monomer-rich product. Values are the average of two biological replicates with error bars representing one standard deviation. The letters next to the bars indicate statistically significant ($p < 0.05$) groupings of the maximum OD_{600} values such that any bars not sharing a common letter significantly differ. Asterisks indicate significance relative to the corresponding emulsifier-only control culture. The stars indicate p-values of ≤ 0.05 (*) and ≤ 0.0001 (****). Growth curves are provided as Fig. S3.

Fig. 5. Emulsification of model phenolic monomers (a) *p*-coumarate, (b) benzoate and (c) vanillin impacts growth. *P. putida* KT2440 was grown for 70 hours at 30 °C and 200 RPM and initial pH of 7.0, and 0.08 g of emulsifier blend per g of monomer was used. Values are the average of two

biological replicates with the error bars representing one standard deviation. The stars indicate p-values calculated by pair-wise Student's two-tailed, equal variance t-tests. The stars indicate p-values of ≤ 0.05 (*), ≤ 0.001 (***), and ≤ 0.0001 (****).

Table 1. Composition analysis of emulsified phenolic monomer-rich product. 1.03 g of the phenolic monomer-rich product was emulsified in 10.0 g DI water with 0.204 g of the 70:30 blend of Tween® 20 and Span® 80 and immediately characterized by GC/FID. A full chromatogram is provided as Fig. S1.

(a) Calibrated compounds detected in the emulsion. Values are the average concentration with the associated standard deviation. Monomers are ordered by concentration.

Compound	CAS #	Concentration (mg/L)
3',5'-dimethoxy-4'-hydroxyacetophenone (acetosyringone)	2478-38-8	610±10
2,6-dimethoxyphenol	91-10-1	394.0±0.9
2,5-dimethoxybenzyl alcohol	33524-31-1	333.0±0.2
2-methylphenol (*o*-cresol) 3-methylphenol (*m*-cresol) 4-methylphenol (*p*-cresol)	95-48-7 108-39-4 106-44-5	272.0±0.6
4-vinylphenol	2628-17-3	264±8
2-methoxy-4-vinylphenol (4-vinylguaiacol)	7786-61-0	261.0±0.4
3-ethylphenol	620-17-7	220.0±0.3
2,5-dimethylphenol	95-87-4	198±1
4-ethoxystyrene (p-vinylphenetole)	5459-40-5	190.00±0.07
3-methoxy-5-methylphenol	3209-13-0	182±0.000
2-methoxy-4-propylphenol (dihydroeugenol)	2785-87-7	180.0±0.2
phenol	108-95-2	170.0±0.7
anisole	100-66-3	160.0±0.2
1,3-dimethylbenzene (*m*-xylene)	108-38-3	152±1
ethylbenzene	100-41-4	151±1
2-methoxy-4-(2-propenyl)phenol (eugenol)	97-53-0	150.0±0.3
1,2-dimethylbenzene (*o*-xylene)	95-47-6	149.0±0.5
2,3-dimethoxytoluene (3-methylveratrole)	4463-33-6	147.0±0.3
2-methylanisole	578-58-5	114.0±0.2
2,6-dimethylphenol	576-26-1	99.0±0.1
3,4-dimethylphenol	95-65-8	85.00±0.06
1,2,3-trimethoxybenzene	634-36-6	78.0±0.5
3,5-dimethylphenol	108-68-9	26.0±0.3
3,4-dimethoxytoluene	494-99-5	23.0±0.3
4-ethyl-2-methoxyphenol (4-ethylguaiacol)	2785-89-9	15.0±0.3
styrene	100-42-5	13.00±0.06
3-methylanisole	100-84-5	5.0±0.2

(b) Calibrated compounds not detected in the emulsion.

	CAS #		**CAS #**
furan	110-00-9	guaiacyl acetone	2503-46-0
indene	95-13-6	3-furan methanol	4412-91-3
benzene	71-43-2	2-methoxyphenol	90-05-1
p-xylene	106-42-3	2,4-dimethylphenol	105-67-9
isoeugenol	97-54-1	1,2-benzenedimethanol	612-14-6
naphthalene	91-20-3	5-hydroxymethylfurfural	67-47-0
levoglucosan	498-07-7	methyl cyclopentenolone	80-71-7
hydroquinone	123-31-9	3'4'-dimethoxyacetophenone	1131-62-0
sinapaldehyde	4206-58-0	2-methoxy-4-methylphenol	93-51-6
2-methylfuran	534-22-5	1,3-benzenediol (resorcinol)	108-46-3
2-furaldehyde	98-01-1	4-allyl-2,6-dimethoxyphenol	6627-88-9
coniferaldehyde	458-36-6	2',4'-dimethoxyacetophenone	829-20-9
sinapyl alcohol	537-33-7	4-methyl-2,6-dimethoxyphenol	6638-0-57
furfuryl alcohol	98-00-0	4'-hydroxy-3'-methoxyacetophenone	498-02-2
m-tolualdehyde	620-23-5	2,6-dihydroxy-4'-methoxyacetophenone	7507-89-3
4-methylanisole	104-93-8		

Table 2. Phenolic monomer recovery is directly related to the amount of emulsifier used. Emulsions were made at room temperature with 70:30 Tween® 20 and Span® 80 blend by vortexing for 5 minutes. Monomer recovery was assessed by the difference in the initial mass of phenolic monomer-rich product and the mass of material remaining after emulsification. Values reported are the averages of at least two replicates ± one standard deviation.

Emulsifier dosage (g of emulsifier blend per g of phenolic monomer-rich product)	0.0136±0.0005	0.055±0.008	0.076±0.002	0.205±0.008
Recovery of phenolic monomer-rich product in emulsion (wt%)	7.400±0.003	10.400±0.005	14.80±0.03	20.700±0.009

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Figure 1

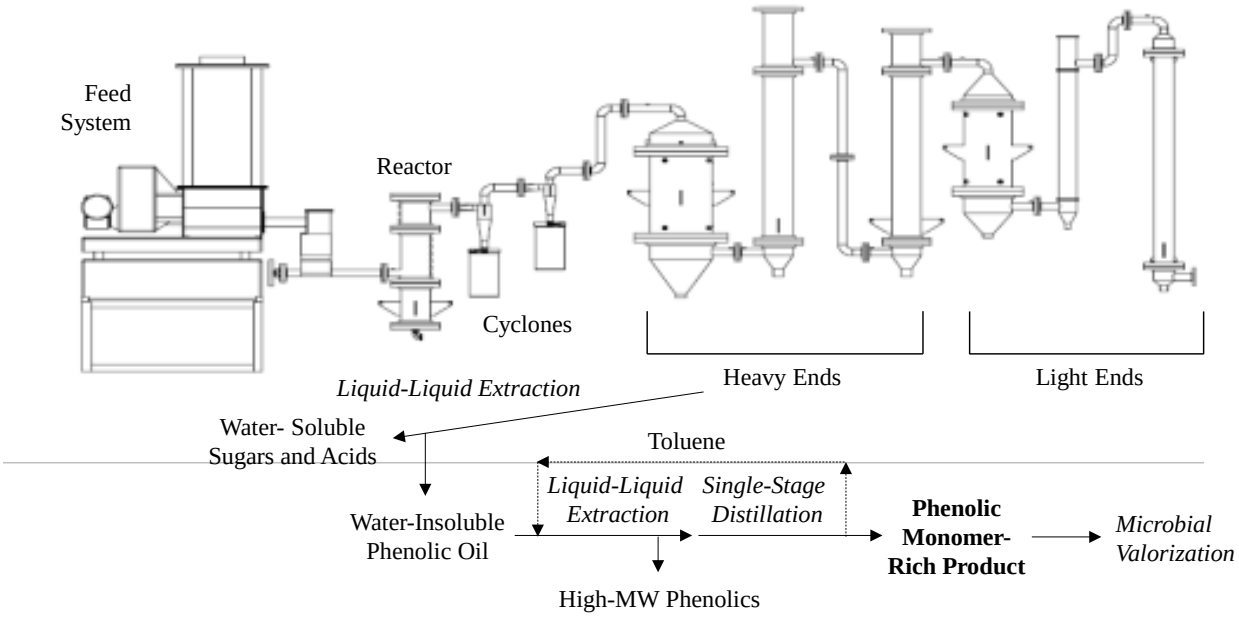
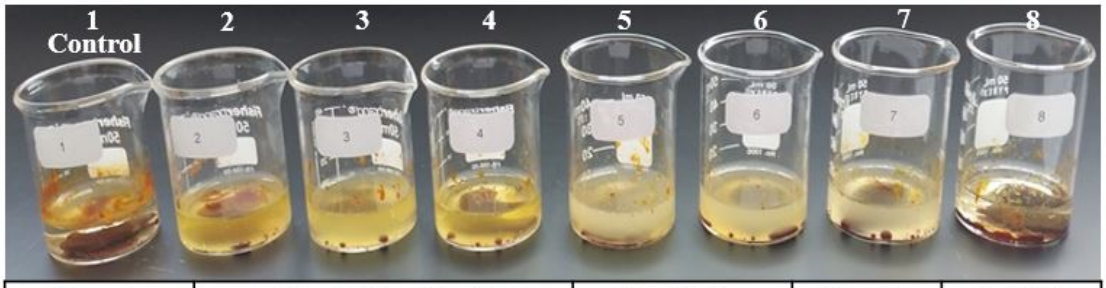
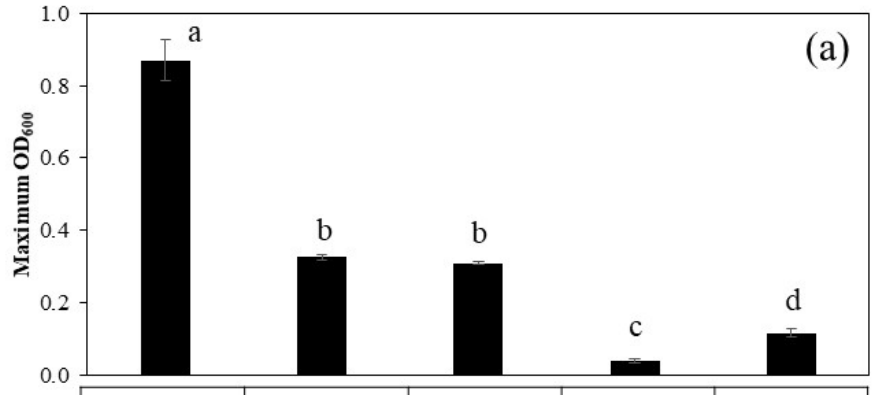

Feed System
Reactor
Cyclones
Heavy Ends
Light Ends
Liquid-Liquid Extraction
Water- Soluble Sugars and Acids
Toluene
Liquid-Liquid Extraction
Single-Stage Distillation
Water-Insoluble Phenolic Oil
Phenolic Monomer-Rich Product
Microbial Valorization
High-MW Phenolics

Figure 2



Sample Number	Emulsifier Blend Composition (wt%)		Emulsifier Blend Calculated HLB	Clarity	Stability
	Tween® 20	Span® 80			
1	0	0	n/a	clear	n/a
2	100	-	16.7	cloudy	too stable
3	86.1	13.9	15.0	cloudy	too stable
4	71.5	28.5	13.2	acceptable	acceptable
5	47.6	52.4	10.2	cloudy	acceptable
6	29.6	70.4	7.97	cloudy	n/a
7	7.30	92.7	5.20	cloudy	n/a
8	-	100	4.30	clear	n/a

Figure 3



Surfactant	Tween®20 + Span®80 (73.9:26.1)	Tween®20 + Span®80 (74.2:25.8)	Tween®20 Only	Tween®20 Only	Span®80 Only
Surfactant dose: g per g of monomer-rich product	0.211	--		0.158	0.0604
wt% of phenolic monomer added to growth medium	0.24	0		0.24	
Estimated wt% of surfactant added to growth medium	0.28	0.25		0.21	0.11

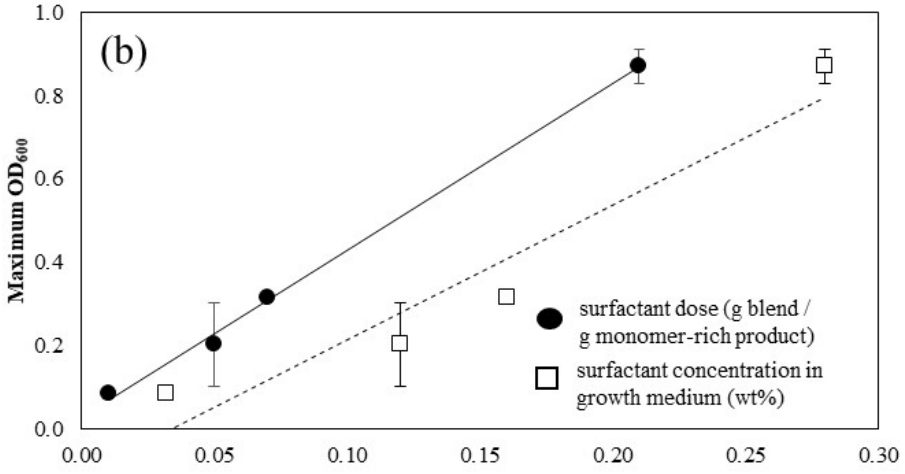
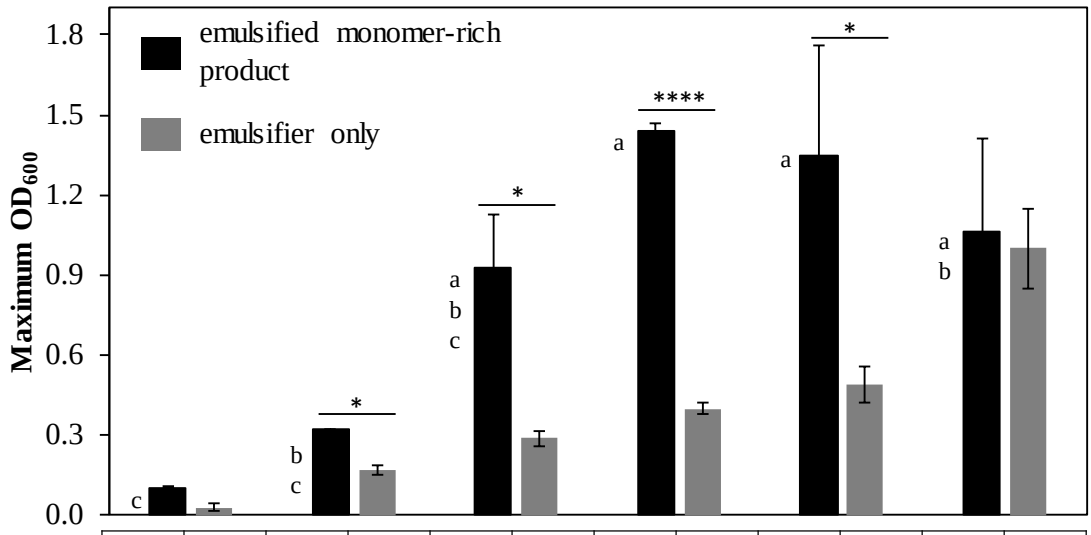
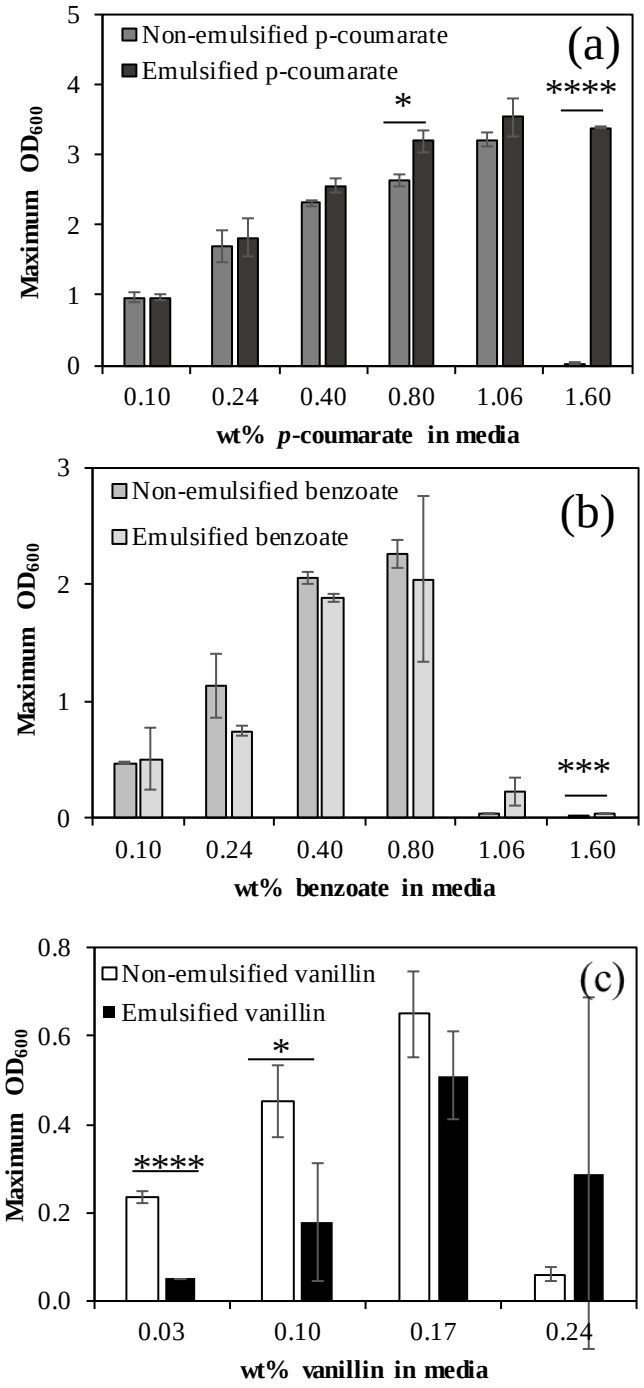


Figure 4



wt% of phenolic monomer in growth medium	0.10	0	0.24	0	0.40	0	0.80	0	1.06	0	1.60	0
estimated wt% of surfactant in growth medium	0.03		0.17		0.29		0.36		0.49		0.82	0.87

Figure 5


(a)
Non-emulsified p-coumarate
Emulsified p-coumarate
*
****
Maximum OD600
0.10
0.24
0.40
0.80
1.06
1.60
wt% p-coumarate in media
(b)
Non-emulsified benzoate
Emulsified benzoate
***
Maximum OD600
wt% benzoate in media
(c)
Non-emulsified vanillin
Emulsified vanillin
****
*
Maximum OD600
0.03
0.10
0.17
0.24
wt% vanillin in media